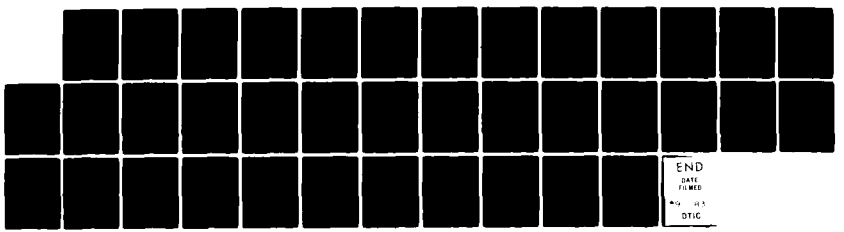
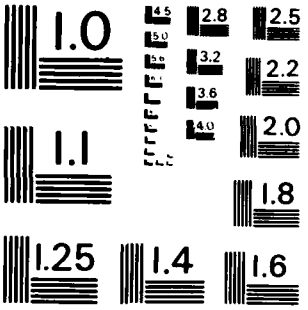
AD-A131 073 EFFECTS OF TRYPANOCIDAL DRUGS ON THE FUNCTION OF TRYPANOSOMES(U) COLORADO STATE UNIV FORT COLLINS DEPT OF PATHOLOGY G C HILL SEP 80 DAMD17-74-C-4046 1/1

UNCLASSIFIED F/G 6/15 NL





AD

EFFECTS OF TRYPANOCIDAL DRUGS ON THE FUNCTION OF TRYPANOSOMES

Annual Progress Report

George C. Hill

September 1980

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-[illegible]

Colorado State University
Fort Collins, Colorado 80523

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. A131072	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Effects of Trypanocidal Drugs on the Function of Trypanosomes		5. TYPE OF REPORT & PERIOD COVERED Annual Progress Report 1 January, 1980-1 Sept., 1980
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) George C. Hill		8. CONTRACT OR GRANT NUMBER(s) DAMD-17-74-C-4046
9. PERFORMING ORGANIZATION NAME AND ADDRESS Colorado State University Department of Pathology Fort Collins, CO 80523		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62770A 3M162770A802.00.051
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE September 1980
		13. NUMBER OF PAGES 37
14. MONITORING AGENCY NAME & ADDRESS(If different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Same as report

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Cultured-infective trypomastigotes; T. rhodesiense; T. brucei; T. gambiense; berenil; suramin; L-α-GP-oxidase; macromolecular synthesis.

alpha

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

During the past twelve months, we have continued our research efforts on the biochemistry of African trypanosomes. We have characterized the in vitro system for growing cultured-infective trypomastigotes of Trypanosoma rhodesiense, T. gambiense, and T. brucei. We have measured a decrease in the glucose levels in these cultures as the organisms increase in cell numbers and have completed a study on the electron transport system present, demonstrating that the α-glycerophosphate oxidase is the only terminal oxidase functioning

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE
S/N 0102-014-6601

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

in cultured-infective trypomastigotes. In determining the nutritional requirements of the cultured-infective trypomastigotes, we have decreased the levels of fetal bovine serum from 20% to 10% and successfully substituted horse serum or new-born calf serum in our cultures. Recently, it has been possible to study the transformation of T. brucei cultured-infective trypomastigotes to procyclic trypomastigotes and a characterization of this system has been initiated.

In examining the capability of African trypanosomes to undergo mitochondrial and cytoplasmic protein synthesis, we have rigorously characterized both systems and identified important biochemical differences between the protein synthesis system in eukaryotic cells and in African trypanosomes. Extremely important is the fact that the mitochondrial protein synthesis system in African trypanosomes is not completely sensitive to the usual inhibitors of mitochondrial protein synthesis, as had been reported by other investigators.

Progress has been made in purifying the α-glycerophosphate oxidase from T. brucei bloodstream trypomastigotes. It is very clear that ubiquinol-1 will be extremely important in identifying the biochemical properties of this terminal oxidase in bloodstream trypomastigotes. In addition, we have conclusively demonstrated that after transformation, procyclic trypomastigotes of T. brucei as well as T. rhodesiense have a branched electron transport system, retaining the α-glycerophosphate oxidase which previously had been thought by other investigators to have been absent in procyclic trypomastigotes.

Our efforts during the next year will concentrate on a characterization and improvement of the in vitro culturing system for T. rhodesiense bloodstream trypomastigotes and the application of this system to the detection of trypanocidal activity in various compounds.

Accession For	
NTIS GRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A	

ABSTRACT

During the past twelve months, we have continued our research efforts on the biochemistry of African trypanosomes. We have characterized the *in vitro* system for growing cultured-infective trypomastigotes of *Trypanosoma rhodesiense*, *T. gambiense*, and *T. brucei*. We have measured a decrease in the glucose levels in these cultures as the organisms increase in cell numbers and have completed a study on the electron transport system present, demonstrating that the α-glycerophosphate oxidase is the only terminal oxidase functioning in cultured-infective trypomastigotes. In determining the nutritional requirements of the cultured-infective trypomastigotes, we have decreased the levels of fetal bovine serum from 20% to 10% and successfully substituted horse serum or newborn calf serum in our cultures. Recently, it has been possible to study the transformation of *T. brucei* cultured-infective trypomastigotes to procyclic trypomastigotes and a characterization of this system has been initiated.

In examining the capability of African trypanosomes to undergo mitochondrial and cytoplasmic protein synthesis, we have rigorously characterized both systems and identified important biochemical differences between the protein synthesis system in eukaryotic cells and in African trypanosomes. Extremely important is the fact that the mitochondrial protein synthesis system in African trypanosomes is not completely sensitive to the usual inhibitors of mitochondrial protein synthesis, as had been reported by other investigators.

Progress has been made in purifying the α-glycerophosphate oxidase from *T. brucei* bloodstream trypomastigotes. It is very clear that ubiquinol-1 will be extremely important in identifying the biochemical properties of this terminal oxidase in bloodstream trypomastigotes. In addition, we have conclusively demonstrated that after transformation, procyclic trypomastigotes of *T. brucei*, as well

as T. rhodesiense, have a branched electron transport system, retaining the α-glycerophosphate oxidase which previously had been thought by other investigators to have been absent in procyclic trypomastigotes.

Our efforts during the next year will concentrate on a characterization and improvement of the in vitro culturing system for T. rhodesiense bloodstream trypomastigotes and the application of this system to the detection of trypanocidal activity in various compounds (1,2).

APPROACH TO THE PROBLEM

As we have noted in previous annual reports, our approach to the problem of developing effective trypanocidal drugs is to study two specific and related areas of the biochemistry of trypanosomes, nuclear and mitochondrial gene expression. The interrelationships of these two systems can be seen in Figure 1. In addition, we are concerned with the regulation and control of the functioning of the terminal oxidases in bloodstream trypomastigotes. In order to identify targets for potential trypanocides, we must learn more about the properties of the mitochondrion in trypanosomes including the replication and transcription of kDNA and the repression and synthesis of mitochondrial electron transport systems. We also need to study the properties of the α-GP oxidase system. If we can alter the functioning of the mitochondrion or other essential electron transport systems in trypanosomes, we should be able to inhibit the continuation of the life cycle of the trypanosome.

We are also interested in the inhibition of novel enzymes on processes which are under control of the nuclear genome. These include processes such as antigenic variation or synthesis of L-α-GP oxidase on cytoplasmic ribosomes. Our goal in the development of novel trypanocidal drugs is to gain additional knowledge on the control mechanisms involved in the transcription of nuclear and mitochondrial genes in these organisms. In this way, we hope to identify potential targets for trypanocides that so far have not been investigated. The proper establishment of the primary mode of action of a drug requires a systematic study of its effect on the various metabolic processes of the cell at the lowest concentrations that inhibit growth. Only when such a survey is complete can it be concluded confidently that a particular pathway or reaction is most sensitive to inhibition by a drug and is therefore the primary target of that drug. We are carrying out this type of systematic study with

several trypanocidal drugs including berenil, suramin, antrycide and ethidium bromide. These types of studies have only rarely been performed with trypanocidal drugs.

The numerous and necessary speculative points raised in any discussion of trypanocidal drug action are now mostly capable of experimental verification with currently available techniques and data from related fields of cell and molecular biology and biochemistry. Given the necessary attention, this largely neglected but important field should yield results of considerable value, not only for an understanding both of trypanocidal drug action and of trypanosomal metabolism, but for cell biology in general. This is the purpose of this contract. So far as trypanocidal drug design is concerned, we have previously noted that the era of intelligent empericism is unlikely to be superseded until the balance of effort and expenditure on drug production is adjusted more favorably in the direction of research on the metabolism of trypanosomes and on the mechanisms whereby existing drugs exert their specific effects.

In developing new trypanocidal agents, we are investigating:

1. Effects of trypanocidal drugs affecting enzyme systems in trypanosomes as well as the host tissues;
2. Comparisons of homologous enzymes in host and trypanosomes;
3. Unique cell components or metabolic pathways in trypanosomes;
4. The basis of the selective toxicity of known drugs.

BACKGROUND

African trypanosomiasis is confined to Africa by the distribution of its vectors. T. gambiense infection occurs over a broad belt from Senegal in the west to the great lakes Victoria, Albert, Benguelo, and Tanganyika in the east, extending as far south as north Angola. T. rhodesiense is located in east and central Africa and is scattered over a longitudinal band from the south Sudan to Mozambique. The importance of trypanosomiasis of man and animals in Africa has been summarized recently by Kershaw (3) in the statement:

> "The World Health Organization in determining the ten major health problems facing mankind places trypanosomiasis of man and his domestic animals high on the list along with malaria, cancer and heart diseases."

Identification of New Trypanocidal Drugs

The need for new trypanocides cannot be overemphasized. At present, chemotherapy of African trypanosomiasis is dependent on a relatively small number of synthetic drugs. Suramin and pentamidine are used for prophylaxia and treatment of early stages of the disease in man. Organic arsenicals such as tryparsamide and melaminyl compounds are used for advanced cases when trypanosomes have invaded the central nervous system. The disease in cattle and other domestic animals is controlled by quaternary ammonium trypanocides (e.g., antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. As pointed out recently by Newton (4), resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to another.

In human trypanosomiasis, there is still an urgent requirement for a cheap, simply administered and well-tolerated, preferably "one-shot" drug which would be as effective a prophylactic as pentamidine and active therapeutically against all stages of the infection in both Gambian and Rhodesian sleeping sickness. It should also be incapable of inducing drug resistance and active also against strains with acquired resistance to other drugs.

Possibly the two requirements of prolonged tissue retention (for prophylaxis) and ability to penetrate into the central nervous system are mutually exclusive, but with increasing knowledge of the structure and function of the so-called "blood-brain barrier," this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review on the mode of action of trypanocidal drugs has been prepared by Williamson (5). More recent studies have suggested that berenil and ethidium bromide form complexes with DNA. In the case of ethidium, it is clear this drug is a potent and selective inhibitor of DNA synthesis. It has been shown by several investigators that both phenanthridines and acridines combine with DNA by the heterocyclic chromophore of the drug molecules becoming inserted, or intercalated, between the adjacent base pairs in the double-stranded helix of DNA. Such intercalation is achieved by a partial uncoiling of the DNA helix which results in the base pairs above and below the bound drug molecule becoming separated by twice their normal distance (4).

More recently, it has been shown that phenanthridines also bind to supercoiled DNA of the type found in certain tumor viruses, mitochondria

of many cell types and kinetoplast of trypanosomes. There is evidence that these drugs bind preferentially to such DNA *in vivo* and give rise to dyskinetoplastic trypanosomes (6) and "petite mutants" of yeast (7). The molecular basis of this preferential binding is not yet fully understood. The findings that have been observed could adequately explain the growth inhibitory activity of phenanthridine drugs but it remains to be established whether their primary action on bloodstream forms of trypanosomes is to inhibit DNA synthesis.

Berenil, an aromatic diamidine, has been shown to interact with DNA and can selectively block kinetoplast replication (8, 9). The earliest reported effect observed of berenil is the localization in the kinetoplast of *T. brucei*. This has been detected by ultraviolet microscopy with an hour of a curative dose being injected intraperitoneally into infected mice and within seconds of the drug being added to an *in vitro* suspension of trypanosomes (9). Recent work has shown that berenil can form complexes with purified DNA, but in contrast to phenanthridines, there is good evidence that the complexes are not formed by intercalation (10).

A detailed examination of kinetoplast DNA isolated from berenil-treated *T. cruzi* has shown that many of the small circular DNA molecules appear as branched structures (8). These forms, which are thought to be replicative molecules, are rarely seen in control preparations, suggesting that berenil does not block the replication of kinetoplast DNA at initiation but binds preferentially to certain specific points in the circular DNA molecule. As for phenanthridines, it cannot be said what is the primary effect of berenil or other diamidines on trypanosomes.

The mode of action of suramin remains enigmatic even after more than a half century of use. In vitro exposure to trypanosomes to suramin at concentrations as low as 10^{-5} M is known to reduce their infectivity whereas concentrations as high as 10^{-2} M do not affect the motility or respiration of cells. As would be expected from its structure, the drug binds avidly to basic proteins and is known to inhibit many isolated enzymes (5). The most sensitive enzymes examined appear to be hyaluronidase, inhibited at 10^{-5}-10^{-6} M, fumarase, inhibited at ca. 10^{-7} M, urease at pH 5 (ca. 10^{-4} M), hexokinase (10^{-4}-10^{-5} M), and RNA polymerase (10^{-5} M) (11). Recent studies by our laboratory supported by this contract (12) and other investigators (13, 14) have demonstrated that suramin also inhibits the L-α-GP oxidase in bloodstream trypanosome in vitro. Whether this is its mode of action in vivo remains to be determined.

The ready absorption of this drug by plasma proteins may well account for the long retention time of the compound in man and animals and contribute to its value as a prophylactic agent. The question of how a molecule as large as suramin enters trypanosomes is an interesting one and it seems possible that, when protein bound, suramin actively stimulates pinocytosis. As with the other drugs that we have discussed, there is evidence that suramin becomes localized in lysosomes. Again whether this is important to the trypanocidal action of the drug or whether it is a secondary phenomenon is unknown.

The Importance of African Trypanosomiasis as a Public Health Problem

The WHO recently published information on the importance of African trypanosomiasis as a public health problem. Human trypanosomiasis, causing sleeping sickness, and animal trypanosomiasis, referred to as nagana, affecting cattle and other domesticated animals, are the two classical notorious plagues

of Africa rooted in the continent since time immemorial. Sleeping sickness constitutes a permanent and serious risk to the health and well-being of at least 35 million people, and animal trypanosomiasis is the main obstacle to the development of the vast potential for livestock production in the continent. Any involvement of our military troops in areas endemic with African trypanosomiasis would be disastrous.

Ten thousand new cases of human trypanosomiasis are known to occur yearly, but this figure does not truly reflect the importance of the disease as a public health problem. As with many tropical diseases, prevalence figures are underestimates due to failure to recognize the disease and to under-reporting. The relatively low prevalence is due to the major control efforts which have been made over the past 50 years. Without these, sleeping sickness would still be a major cause of death, as it was at the turn of the century, with great epidemics raging along the Congo river and the northern shores of Lake Victoria costing the lives of some 750,000 people.

At the present time, some 10 million people at risk are examined annually by mobile teams at an estimated cost of 5 million dollars. Expenditure for control of tsetse flies is at least ten times higher, but the total is difficult to evaluate since most efforts are directed towards control related to animal disease. In view of the potential serious danger of sleeping sickness, national health services accord high priority to control services but efforts are frequently inadequate since sufficient resources in terms of finance, manpower and administrative facilities may not be available.

An outbreak of sleeping sickness is a dramatic event in a community since the disease causes severe symptoms due to lesions of the central nervous system and is fatal if not treated. Outbreaks may cause populations to

abandon villages and fertile farmlands, and the effect is such that even after two generations, fear of re-exposure may prevent the people from returning. Current control measures do not usually eliminate the disease; moreover, they are costly and cumbersome. With the available tools, control is a continuing effort, producing suppression rather than eradication. The experience of the past 50 years has been that whatever control efforts are interrupted, for example due to political or economic circumstances, or out of complacency, a flare-up of the disease will sooner or later occur.

A recent example was the resurgence of trypanosomiasis in Zaire in the early 1960's, when after six years of interruption of surveillance, prevalence figures rose from 0.01% to 12%, and even to 18% in some areas. Outbreaks of unknown severity are now reported from Angola, Cameroon and the Sudan. It is to be expected that more outbreaks will occur in coming years unless improved control measures can be found. Development of new tools is therefore a matter of urgency, not only as a means to eradicate the disease but to provide measures which are more effective and can be more widely applied than those presently available.

RESULTS AND DISCUSSION

A. Cultivation of Trypanosomes in Vitro

During the past year, we have concentrated on growing and maintaining cultured-infective trypomastigotes of Trypanosoma rhodesiense EATRO 1895 and T. gambiense TREU 1309. These experiments have been most successful with T. rhodesiense EATRO 1895, and a manuscript was prepared and presented at the Janssen Symposium on the Biochemistry of Parasites and Host-Parasite Relationships in July. We have been able to successfully maintain T. rhodesiense EATRO 1895 over several types of tissue culture cells including Chinese hamster lung tissue culture cells (ATCC CCL 16 Don). We have also grown this strain on New Zealand white rabbit kidney, Rhesus monkey kidney, and embryonic bovine trachea tissue culture cells (EBTr), all available from ATCC. In our initial studies, the trypanosomes were grown using 20% fetal bovine serum. However, it is now possible to substitute either 10% fetal bovine serum or 10% newborn calf serum with the EBTr cells or 10% horse serum with all the tissue culture cells.

Studies have been performed in order to determine the amount of glucose consumption during the growth of T. brucei cultured-infective trypomastigotes in vitro. As can be seen from Table I, when the cultures were initiated at 1.0×10^6 trypanosomes/ml, 24 hours later, a 10% decrease in the glucose concentration was observed as the population increased to 2.9×10^6 trypanosomes/ml. One-third of the medium was removed and 24 hours later there was a 45% reduction in the glucose level as the cell population

increased to 3.0×10^6 cells/ml. A similar change occurred from day 2 to day 3. This suggests that there is a high rate of glucose consumption when significant numbers of trypanosomes are growing and that it may be possible to maintain the cultures by controlling the glucose levels in the growth medium. At the present time, we are evaluating other components in order to see if there are similar changes.

Some experiments have been performed in order to determine if trypanosomes can be grown in chambers where they are separated by a membrane from the tissue culture cells. We have obtained some parabiotic chambers from Bellco (Vineland, New Jersey). In initial studies during these experiments, it has not been possible to grow either T. brucei or T. rhodesiense cultured-infective trypomastigotes under such conditions where they are physically separated from the tissue culture cells. The trypanosomes continue to grow as cultured-infective trypomastigotes for several days and then undergo transformation to procyclic trypomastigotes even though they are growing at 37° C. In addition, the chambers that have been used in these earlier experiments are not ideal for these studies since it is difficult to evaluate them under the inverted microscope. More appropriate chambers are now being designed for these studies and the experiments will be continued.

One of the major problems that we have had during the past year has been contamination of the cultures with yeast or bacteria. For four to five months during the past year (February through June) it has been impossible to do any of our culturing work because of this contamination problem. Regardless of the antibiotic that we used, it was not possible to

eliminate the bacteria or yeast until they were properly identified at our diagnostic laboratory and the essential antibiotics added. In addition, the increase in cost of fetal bovine serum, from $35 per 500 ml to $165 per 500 ml has meant that we must try to identify substitutes for fetal bovine serum. This has been successful, and now with the elimination of contamination in our cultures, we can continue our experiments. However, this has led to a significant delay in our research.

We are also investigating the biochemical properties of the trypanosomes cultured in vitro. It is clear that the predominant oxidase is the α-glycerophosphate oxidase. A detailed discussion on the electron transport system present in T. rhodesiense cultured-infective trypomastigotes, as well as the studies that we have done comparing this electron transport system with other bloodstream trypomastigotes as well as procyclic trypomastigotes is presented in the manuscript entitled: Biochemical studies using Trypanosoma rhodesiense cultured-infective trypomastigotes by George C. Hill which was presented at The Janssen Symposium on the Biochemistry of Parasites and Host-Parasite Relationships (19).

One of the important goals that we made when we began our studies on culturing and maintaining T. rhodesiense EATRO 1895 cultured-infective trypomastigotes was to evaluate the usefulness of this system as a tool in the development of new trypanocides. As outlined in our manuscript, this system is now available. Its development is important since in vitro experiments to test drug sensitivity conducted previously in several laboratories have used either procyclic trypomastigotes or bloodstream trypomastigotes that have been maintained in vitro for several hours. In the

case of procyclic trypomastigotes, it is very clear that the biochemistry of these organisms is markedly very different from bloodstream trypomastigotes. Thus, to assay drugs against procyclic trypomastigotes is not a very reliable system for detecting the action of compounds against the target organism. In addition, it can be extremely deceiving to find trypanocidal activity against procyclic trypomastigotes. In other systems, the use of bloodstream trypomastigotes also has limitations. Some of the in vitro systems in which these organisms have been maintained have not supported growth for any significant period of time. Thus the trypanosomes are in a "dying" system and difficulty interpreting the results can be encountered. It has been possible to detect trypanocidal activity in various compounds using in vitro systems where mice are infected with trypanosomes and then various compounds assayed. However, it is difficult to determine the mode of action of the trypanosomes in this situation. Clearly, the evaluation of the effectiveness of this in vitro system for drug evaluation is important.

B. Growth of Cultured-Infective Trypomastigotes and Procyclic Trypomastigotes of WRATat Strains of Trypanosoma rhodesiense.

During the past twelve months we have attempted to grow cultured-infective forms of Trypanosoma rhodesiense WRATat strains. This has met with some limited success. As can be seen in Figure 2, cultured-infective forms of this strain were obtained. However, the organisms are very difficult to maintain as cultured-infective forms and a decrease in growth occurs in this system which had not occurred in culturing other bloodstream trypomastigotes. The difficulty in growing T. rhodesiense WRATat strains is similar to a difficulty we have observed with other recently isolated strains of T. brucei, T. gambiense and T. rhodesiense. It is possible that this difficulty is associated with the type of tissue culture cells on which the organisms are being grown as well as a need for some additional nutritional requirements. Experiments are continuing to prolong the growth (e.g., 4-5 months) of WRATat strains of T. rhodesiense on tissue culture cells.

Experiments studying the transformation of T. rhodesiense WRATat strains from bloodstream trypomastigotes to procyclic trypomastigotes have been more successful. Transformation is complete in 96 to 120 hours and the organisms reach a cell density of 2-3 x 10^7/ml. These transformation experiments have been performed as described in one of our recent publications in which we studied differentiation of T. brucei LUMP 1026 from bloodstream to procyclic trypomastigotes (15). However, although morphological transformation is complete after 4 days, a much longer period of time is required before the procyclic trypomastigotes have the electron

transport system expected of established procyclic trypomastigotes. The cyanide sensitivity in the absence of SHAM does not reach a plateau level until day 16 to day 20. In addition, antimycin A sensitivity did not reach the maximal level expected in established procyclic trypomastigotes (60-70% inhibition) until 18-22 days. The reasons for the delay in the complete establishment of the electron transport system as would be expected in established procyclic trypomastigotes remains to be determined. However, it is clear with all of the bloodstream African trypanosomes that we have used that the biochemical changes that occur are not as rapid as morphological changes (15).

In addition, we have evaluated the loss of the surface coat during transformation of T. rhodesiense WRATat strains. As can be seen in Table II, on day 0, a positive reaction occurs between the trypanosomes and the monoclonal antibodies, in this particular experiment specific for T. rhodesiense WRATat 3. There is a rapid loss in the surface coat so that on day 5 most of the cells are negative. Electron micrograph studies of trypanosomes during transformation provide a similar result.

During the past year it has been extremely difficult to work with procyclic trypomastigotes because of a significant contamination problem in our cultures with mycoplasma. Because of the shortage of fetal bovine serum, there seems to have been a marked decrease in quality control on different batches of fetal bovine serum. Many vendors have been providing fetal bovine serum which has not been carefully checked for mycoplasma and we have had our research delayed by mycoplasma contamination in our cultures. However, we have now identified the mycoplasma present, Acholeplasma

laidlawii, added proper antibiotics in order to eliminate it from the cultures, and filtered and tested the fetal bovine serum in order to be sure that no mycoplasma contamination is present. However, the lack of fetal bovine serum which has been prepared under the former excellent conditions has delayed our research with procyclic trypomastigotes.

Experiments studying mitochondrial biogenesis during transformation of T. brucei LUMP 1026 from bloodstream trypomastigotes to established procyclic trypomastigotes have also progressed. These studies have shown that the early part of the mitochondrial electron transport chain, inhibited by antimycin A develops prior to the cyanide sensitive portion. A low amount of antimycin A sensitivity (10-20%) is seen after 23-48 hours in culture whereas equivalent amounts of cyanide sensitivity are not observed until 5 days in culture. A normal respiration pattern, typical of established procyclic trypomastigotes, is not seen until about 20 days in culture. Concomitant with these changes in inhibition are changes in the percent stimulation of oxygen utilization used in the artificial electron donor ascorbate-TMPD. No stimulation occurs with ascorbate-TMPD at time 0, with low levels of stimulation occurring after 48 hours in culture and increasing to maximal stimulation of oxygen uptake at 22-26 days in culture.

During this period of respiratory chain development, there is also an increase in mitochondrial enzyme activity, particularly succinate dehydrogenase and succinate cytochrome c reductase. Succinate cytochrome c reductase activity increased from no activity at time 0 to 40-50 μ moles/min/mg protein on day 30 with succinate dehydrogenase activity increasing from no activity at time 0 to 140-160 μ moles/min/mg protein between 24 to 32

days in culture. These levels are consistent with what is to be expected from established procyclic trypomastigotes. Although morphological transformation based on observations by light microscopy is complete by 72-96 hours in culture, as can be seen from the above results, biochemical transformation takes place over a longer period of time.

Similar delay occurs in the spectral observation of several cytochromes. No cytochromes are evident on day 0 or day 5. However, cytochromes $\underline{aa}_3$, $\underline{b}$ and $\underline{c}$ are spectrally detectable by day 9 and reach the levels characteristic of procyclic trypomastigotes by day 25. One of the significant questions we are now considering is why the delay occurs in the synthesis of the cytochrome system during transformation from bloodstream trypomastigotes to procyclic trypomastigotes.

C. Identification of Mitochondrial Protein Synthesis in Trypanosoma brucei

In an attempt to identify a mitochondrial translation system in T. brucei, during the past year we have written a manuscript of our results on the effects of various antibiotics on protein synthesis sytem in procyclic trypomastigotes of T. brucei LUMP 1026 (16). Our results demonstrate that the function of cytoplasmic ribosomes in T. brucei in vivo is sensitive to inhibition by not only cycloheximide, but also by high concentrations of D-chloramphenicol, L-chloramphenicol, erythromycin and tetracycline. The inhibition by these latter four compounds is a possible secondary consequence of a primary inhibition of mitochondrial respiration in vivo. In support of this conclusion, antimycin A inhibits mitochondrial respiration and, secondarily, cytoplasmic protein synthesis in vivo, suggesting that mitochondrial oxidative phosphorylation is necessary for cytoplasmic translation in T. brucei. Tetracycline inhibition of cytoplasmic protein synthesis in vivo may also be due to a direct effect on cytoplasmic ribosomes, since this drug inhibits ribosome function in vitro. Low concentrations of D-chloramphenicol, erythromycin and tetracycline do not inhibit clycloheximide-insensitive protein synthesis in vivo, implying the presence of permeability barriers to these drugs in T. brucei. Mitochondrial fractions isolated from T. brucei possess a ribosomal translation system, the function of which is insensitive to cycloheximide but sensitive to inhibition by low concentrations of D-chloramphenicol, erythromycin and tetracycline. The total results indicate that a drug such as D-chloramphenicol should only be used with caution in studies of mitochondrial biogenesis in T. brucei.

Our results clearly show that an examination of total protein synthesis in procyclic trypomastigotes of T. brucei reveals that the majority of the cell translational activity is catalyzed by cycloheximide sensitive cytoplasmic ribosomes. The major interest, however, is the observation that high concentrations of D-chloramphenicol, erythromycin and tetracycline, which typically inhibit mitochondrial ribosomes, also strongly inhibit cytoplasmic protein synthesis in vivo. As we note in the Discussion Section of our manuscript, this effect of D-chloramphenicol and erythromycin would not appear to be due to inhibitory action on cytoplasmic ribosome function directly since these compounds do not inhibit cytoplasmic ribosome activity in vitro at concentrations (700-1500 µg/ml) which are strongly inhibitory in vivo. Rather, a primary inhibition by these drugs of mitochondrial respiration (and hence oxidative phosphorylation and ATP synthesis) would occur over the same concentration ranges that affect protein synthesis, would appear to be a sufficient explanation for the effects of these drugs on total protein synthesis in vivo.

Our results with the in vitro studies reported with the mitochondrial amino acid incorporating system allows us to conclude that the enriched mitochondrial fractions from T. brucei contain a ribosomal translation system, the activity of which is insensitive to inhibition by cycloheximide (3.5mM), but sensitive to a number of inhibitors of bacterial and mitochondrial ribosomal functions such as D-chloramphenicol, erythromycin and tetracycline (0.01 - 1 mM). In this respect, this in vitro mitochondrial translation system resembles in vitro systems described for mitochondria from other eukaryotes. However, differences in the degree of sensitivity of mitochondrial

protein synthesis to antibiotic inhibition may exist between the various systems. For example, the translational activity of mitochondria from T. brucei would appear to be less sensitive to inhibition to D-chloramphenicol compared with the sensitivity exhibited by mitochondrial systems from yeast and rat liver. A more detailed description of the properties of mitochondrial translation system from T. brucei may be possible if conditions can be devised under which mitochondria exhibit the higher specific activity of incorporation than that we have found to date. Our results obtained so far suggest very strongly that a mitochondrial protein synthesis system is present in T. brucei. However, we must continue these studies in order to obtain a mitochondrial preparation which demonstrates a high degree of protein synthesis as well as a high degree of respiratory control. I would refer the reviewers of this proposal to the manuscript entitled: Inhibitory effects of chloramphenicol isomers and other antibiotics on protein synthesis and respiration in procyclic Trypanosoma brucei brucei by T. W. Spithill, S. P. Shimer, and G. C. Hill which shall be published in Molecular and Biochemical Parasitology and is included in the appendix (16).

D. Studies on the α-Glycerophosphate Oxidase

Ubiquinol-1 has been used as an alternate donor to the α-glycerophosphate oxidase in T. brucei bloodstream trypomastigotes. The optimum concentration of ubiquinol-1 required to give a sufficient rate with renografin purified mitochondria was observed to be 0.5 M. The rate obtained with ubiquinol-1 was 4 to 5 times the rate obtained with α-glycerophosphate as substrate.

Recently the preparation of reduced quinol substrate has been prepared by a procedure which not only yields complete reduction but also is applicable to different quinols. This is a modification of a procedure suggested by Peter R. Rich from the Department of Biochemistry at the University of Cambridge in Cambridge, England. A suitable amount of quinone is first dissolved in 50 ml of diethyl ether and placed in a separatory funnel. A solution of 1 gm sodium dithionite in 50 ml of water is then added and the entire solution shaken vigorously until the quinone has been reduced to the colorless quinol. The aqueous layer is discarded and the ether layer is washed twice with saturated salt solution. After filtration through anhydrous sodium sulfate, the ether is dryed off by vacuum dessication. The quinol is then dissolved in a suitable volume of 96% ethanol and stored at -20°C. Complete reduction of ubiquinone can be determined by measuring the spectrum of the ubiquinol. Ubiquinone has an absorption maximum at 275 nm and ubiquinol has an absorption maximum at 290 nm. Other quinones to be reduced and evaluated at artificial electron donors to the α-glycerophosphate oxidase are plastoquinone-1, coenzyme Q_9, coenzyme Q_{10} and 2,3-dimethoxy-5-methyl-6-decyl 1,4-benzoquinone.

In order to isolate mitochondria from T. brucei bloodstream trypomastigotes so that isolation of the α-glycerophosphate oxidase can occur, a procedure had to be adapted for large scale yield. This has been successful by using a modification of the isolation procedure of mitochondria from Leishmania tarentolae that is described by Braly et al. (17). The best yield of mitochondria has been obtained when a crude preparation of mitochondria from approximately 1.0×10^{11} trypanosomes has been centrifuged on a 20-35% renografin gradient. The renografin purified mitochondria were assayed for α-glycerophosphate oxidase activity. The results demonstrated that the purification scheme from the homogenate of the bloodstream trypomastigotes to renografin purified mitochondria allowed a 31% retention of total α-glycerophosphate oxidase activity and an enrichment of the α-glycerophosphate oxidase activity by 27 fold (Table III).

Research in this area has also involved a study of the flow of electrons through the terminal oxidases present in bloodstream and procyclic trypomastigotes of T. brucei LUMP 1026, using salicylhydroxamic acid (SHAM) and cyanide. Respiration in bloodstream trypomastigotes was completely inhibited by 0.5 mM SHAM with a K_i below 10 μM. The K_i for SHAM in procyclic trypomastigotes was 70 μM. In procyclic trypomastigotes, there are at least three terminal oxidases, of which the two major ones are cytochrome aa_3 oxidase, sensitive to cyanide inhibition and α-glycerophosphate oxidase, sensitive to SHAM inhibition. These two oxidases contribute 60 and 30% respectively to total cell respiration. Inhibition of the cytochrome system with cyanide causes an increase in the flow of electrons through the α-glycerophosphate oxidase activity, and inhibition of this terminal oxidase

system with SHAM stimulates electron flow through the cytochrome system. Succinate oxidation in the mitochondrial fraction is partially inhibited by SHAM and thus SHAM sensitive respiration is not inhibited by antimycin A. The kinetic state of respiration by procyclic trypomastigotes fits a model proposed by Bahr and Bonner to determine the maximum rates of two competing electron transport pathways. Thus, we have concluded that the electron transport chain in T. brucei is branched. These results have recently been published in a manuscript entitled: Evidence for a branched electron transport chain in Trypanosoma brucei by Muturi Njogu, Carla Whittaker and George C. Hill which was published in Molecular and Biochemical Parasitology (18).

CONCLUSIONS

Our primary accomplishments during this past year are:

A. Adapted and maintained Trypanosoma rhodesiense WRATat strains in vitro as cultured-infective trypomastigotes for short periods of time;

B. Prepared and had accepted a manuscript on the improved methods for growing and maintaining T. rhodesiense cultured-infective trypomastigotes (19);

C. Prepared and had published a manuscript on the electron transport systems present in T. rhodesiense bloodstream and procyclic trypomastigotes (20);

D. Prepared and had published a manuscript on the branched electron transport system present in T. brucei procyclic trypomastigotes (18);

E. Prepared two manuscripts which have been accepted on biochemical changes that occur during transformation of T. brucei bloodstream trypomastigotes to procyclic trypomastigotes (15,21);

F. Purified from T. brucei the α-glycerophosphate oxidase 27-fold and increased our ability to prepare ubiquinol-1, which will be important in further steps to purify this enzyme;

G. Prepared and had accepted a manuscript detailing the mitochondrial protein synthesis system in T. brucei procyclic trypomastigotes (16).

References

1. Hill, G.C., Shimer, S., Caughey, B. and Sauer, S. 1978. Growth of infective forms of Trypanosoma (T.) brucei on buffalo lung and Chinese hamster lung tissue culture cells. Acta Tropica 35: 201-207.

2. Hill, G.C., Shimer, S., Caughey, B. and Sauer, S. 1978. Growth of infective forms of Trypanosoma rhodesiense in vitro, the causative agent of African Trypanosomiasis. Science 202: 763-765

3. Kershaw, W.E. 1970. Forward. In: The African Trypanosomiasis, pp. XII-IX. Mulligan, H.W. (ed.). (George Allen and Unwin Ltd., London).

4. Newton, B.A. 1974. Chemotherapy of trypanosomiasis and leishmaniasis. Trypanosomiasis and Leishmaniasis with Special Reference to Chagas Disease, pp. 285-307. (Associated Scientific Publishers, New York).

5. Williamson, J. 1970. Review of chemotherapeutic and chemoprophylactic agents. The African Trypanosomiasis, pp. 125-221. Mulligan, H.W. (ed.). (George Allen and Unwin Limited, London).

6. Steinert, M. 1969. Specific loss of kinetoplastic DNA in Trypanosomatidae treated with ethidium bromide. Exp. Cell Res. 55: 148-252.

7. Slonimski, P.P., Perrodin, G. and Croft, J.H. 1968. Ethidium bromide induced mutation of yeast mitochondria. Biochem. Biophys. Res. Commun. 30: 232-239.

8. Brack, C.H., Delain, E., Riou, G., and Festy, B. 1972. Molecular organization of the kinetoplast DNA in Trypanosoma cruzi treated with berenil, a DNA interacting drug. J. Ultra. Res. 39: 568-579.

9. Newton, B. A. and Le Page, R. W. F. 1967. Perferential inhibition of extracellular deoxyribonucleic acid synthesis by the trypanocide berenil. Biochem. J. 105: 50.

10. Waring, J. J. 1970. Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation. J. Mol. Biol. 54: 247-279.

11. Hill, G. C. and Bonilla, C. A. 1974. In vitro transcription of kinetoplast and nuclear DNA in Kinetoplastida. J. Protozool. 21: 632-638.

12. Hill, G. C. 1977. Characterization of the electron transport systems present during differentiation of African trypanosomes. Function of Alternative Oxidases (ed. Degn, J., Lloyd, D. and Hill, G. C.) pp. 67-77, Pergamon Press, Oxford.

13. Evans, D. A., and Brown, R. C. 1973. m-chlorobenzhydroxamic acid - an inhibitor of cyanide-insensitive respiration in Trypanosoma brucei. J. Protozool. 20: 157-160.

14. Clarkson, A., and Brohn, F. 1976. Trypanosomiasis: an approach to chemotherapy by the inhibition of carbohydrate catabolism. Science 194: 204-206.

15. Bienen, E. J., Hammadi, E. and Hill, G. C. 1980. Trypanosoma brucei: Biochemical and morphological changes accompanying in vitro transformation of bloodstream trypomastigotes to procyclic trypomastigotes. Exper. Parasit., accepted, in press.

16. Spithill, T. W., Shimer, S. P. and Hill, G. C. 1981. Inhibitory effects of chloramphenicol isomers and other antibiotics on protein synthesis and respiration in procyclic Trypanosoma brucei brucei. Mol. Biochem. Parasit., accepted, in press.

17. Braly, P., Simpson, L. and Kretzer, F. 1974. Isolation of kinetoplast-mitochondrial complexes from Leishmania tarentolae. J. Protozool. 21:782-790.

18. Njogu, R. M., Whittaker, C. and Hill, G. C. 1980. Evidence for a branched electron transport chain in Trypanosoma brucei. Mol. Biochem. Parasit. 1:13-30.

19. Hill, G. C. 1980. Biochemical studies using Trypanosoma rhodesiense cultured-infective trypomastigotes. In: Biochemistry of Parasites and Host-Parasite Relationships (ed. Van den Bossche, H.) Elsevier/ North Holland, Amsterdam, in press.

20. Hill, G. C. and Degn, H. 1979. Reaction of terminal oxidases with oxygen during differentiation in African trypanosomes. In: Biochemical and Clinical Aspects of Oxygen (ed. Caughey, W.), pp. 405-420. Academic Press, N. Y.

21. Bienen, E. J., Hammadi, E. and Hill, G. C. 1980. Initiation of trypanosome transformation from bloodstream trypomastigotes to procyclic trypomastigotes. J. Parasit., accepted, in press.

FIGURE LEGENDS

Fig. 1. The site of action of various inhibitors on the nucleo-cell sap system and the mitochondrial system.

Fig. 2. Trypanosoma rhodesiense WRATat 3 cultured-infective trypomastigotes growing on embryonic bovine trachea tissue culture cells. The cells had been growing for 20 days. (x 200).

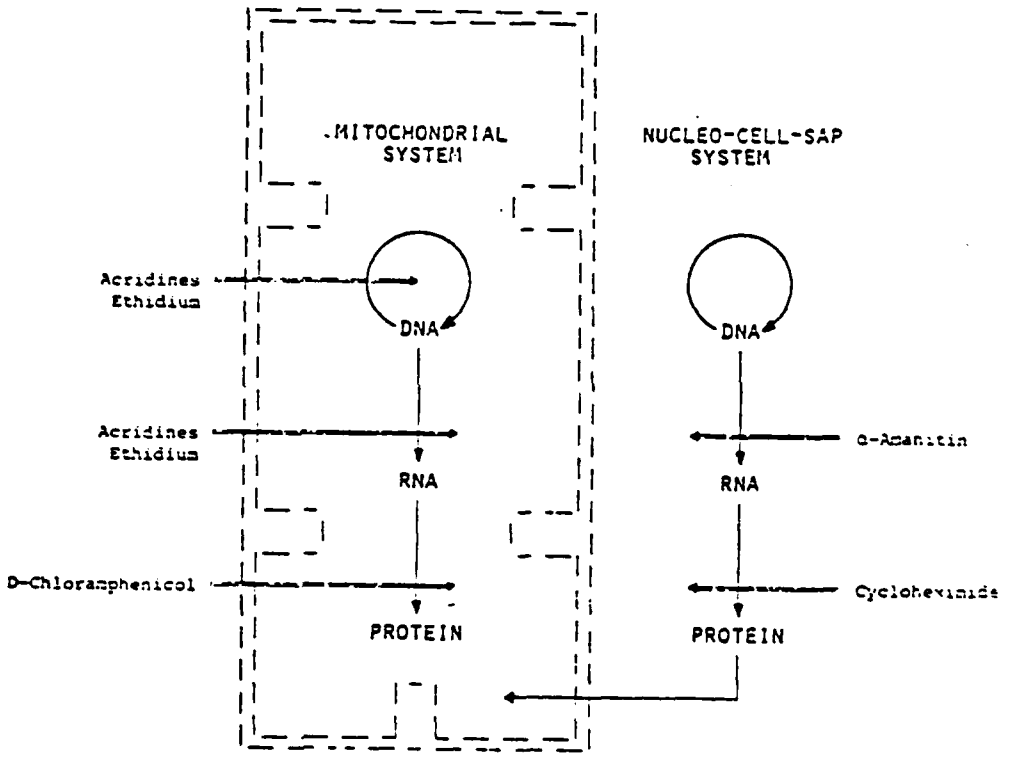

MITOCHONDRIAL SYSTEM
NUCLEO-CELL-SAP SYSTEM
Acridines Ethidium
DNA
DNA
Acridines Ethidium
RNA
α-Amanitin
RNA
D-Chloramphenicol
Cycloheximide
PROTEIN
PROTEIN

Figure 1

Figure 2

TABLE I

Glucose Utilization by Trypanosoma brucei

Cultured-Infective Trypomastigotes

	mg Glucose/100 ml media		Percent Reduction	Trypanosomes/ml
	Fresh media	24 hr later		
T_o	122	110	10	1.0×10^6
T_{24}	137	75	45	2.9×10^6
T_{48}	109	60	45	3.4×10^6

TABLE II

Loss of Surface Antigen During Transformation

of Trypanosoma rhodesiense WRATat 3

	REACTIVITY*	
DAY	ANTIGEN 3	ANTIGEN 5
0	70%	0
2	50%	0
5	10%	0
13	0	0

*Reactivity is scored by brilliance of fluorescence.

Monoclonal antibodies against WRATat 3 and WRATat 5 were produced by Dr. Gary Campbell, University of New Mexico, Albuquerque, New Mexico.

The WRATat strains are recent isolates prepared by the staff at Walter Reed Army Institute, Washington, D.C.

TABLE III

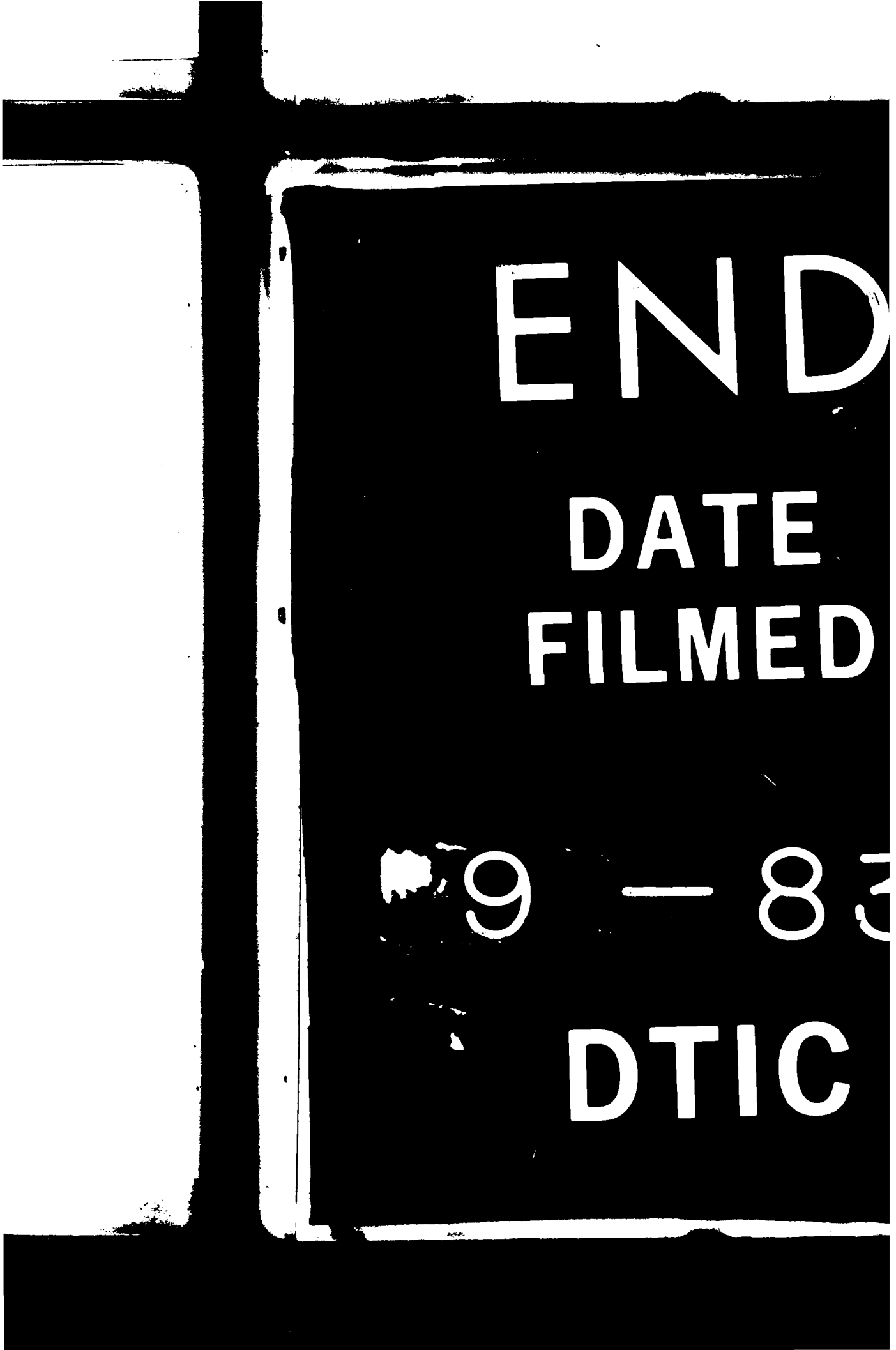
Enrichment of α-Glycerophosphate Oxidase Activity from

Trypanosoma brucei Mitochondria

Bloodstream Trypomastigotes

10.0mM
α-Glycerophosphate

	Homogenate	Pre-DNase I Treated Mitos.	Renografin Purified Mitos.
Units/mg protein	5.0	19.0	137.0
Total volume	100.0 ml	30.0 ml	1.0 ml
Total protein	438.0 mg	204.0 mg	5.0 mg
Total units	2,190.0	3,876.0	685.0
Enrichment	1 fold	4 fold	27 fold
% Yield	100%	177%	31%


END
DATE
FILMED
9 — 83
DTIC